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Ultraviolet fluorescence of coelenteramide and coelenteramide-containing fluorescent proteins. Experimental and theoretical study

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ABSTRACT

Coelenteramide-containing fluorescent proteins are products of bioluminescent reactions of marine coelenterates. They are called 'discharged photoproteins'. Their light-induced fluorescence spectra are variable, depending considerably on external conditions. Current work studies a dependence of light-induced fluorescence spectra of discharged photoproteins obelin, aequorin, and clytin on excitation energy. It was demonstrated that photoexcitation to the upper electron-excited states (260–300 nm) of the discharged photoproteins initiates a fluorescence peak in the near UV region, in addition to the blue-green emission. To characterize the UV fluorescence, the light-induced fluorescence spectra of coelenteramide (CLM), fluorophore of the discharged photoproteins, were studied in methanol solution. Similar to photoproteins, the CLM spectra depended on photoexcitation energy; the additional peak (330 nm) in the near UV region was observed in CLM fluorescence at higher excitation energy (260–300 nm). Quantum chemical calculations by time depending method with B3LYP/cc-pVDZ showed that the conjugated pyrazine-phenolic fragment and benzene moiety of CLM molecule are responsible for the additional UV fluorescence peak. Quantum yields of CLM fluorescence in methanol were 0.028 ± 0.005 at 270–340 nm photoexcitation. A conclusion was made that the UV emission of CLM might contribute to the UV fluorescence of the discharged photoproteins. The study develops knowledge on internal energy transfer in biological structures – complexes of proteins with low-weight aromatic molecules.

1. Introduction

Fluorescent proteins are modern and promising tools for biological and medical investigations. They are applied as fluorescent biomarkers to monitor intracellular processes such as visualization of local protein concentrations, to determine rates of biochemical processes, growth of cell clones including pathogenic bacteria and tumors. Intracellular synthesis is an important benefit of these biomarkers.

Main structural components of fluorescent proteins are aromatic fluorophore and polypeptide. The most known representative of the fluorescent proteins is Green Fluorescent Protein (GFP). It was isolated from jellyfish *Aequorea victoria* by Prof. Shimomura, Nobel Prize Laureate-2008 [1]. A lot of 'colored' fluorescent proteins, homologous to GFP, are known by now [2–4]. A fluorophore of GFP-like proteins is non-external prosthetic group, a product of intracomplex chemical transformation of three aminoacid residuals. Apart from the GFP-like proteins, the class of fluorescent proteins also includes coelenteramide-containing proteins, isolated from luminous marine coelenterates – jellyfishes *Aequorea* [5] and *Phialidium* (*Clytia*) [6], hydroid *Obelia longissima* [7], etc. A fluorophore of the coelenteramide-containing proteins is an external molecule, coelenteramide, which is non-covalently bound to a protein inside its hydrophobic cavity.

Coelenteramide, N-[2-benzyl-6-(4-oxocyclohexa-2.5-dien-1-ylidene)-1H-pyrazin-3-yl]-2-(4-hydroxyphenyl) acetamide, is a heteroaromatic fluorescent compound. Chemical structure of coelenteramide (neutral and ionized forms) is presented in Fig. 1. Aromatic fragments that might be involved to electronic excitation are marked here with letters *F*, *P,* and *B,* corresponding to phenolic, pyrazine, and benzene rings, respectively.

Coelenteramide (CLM) is a photochemically active molecule; it is able to be a proton donor in its electron-excited states, generating several forms of different fluorescent state energy [8] and, hence, different fluorescence color. Fig. 1 shows schematically the different fluorescent state energies of neutral and ionized (or partly ionized) CLM forms. Contributions of the forms to visible fluorescence spectra depend on efficiency of the photochemical process (Fig. 1) and are governed by CLM microenvironment in proteins or solutions [9–14].

First CLM-containing fluorescent protein was isolated and studied by Prof. Shimomura, simultaneously with GFP. It was called as 'Blue Fluorescent Protein' [5]. As opposed to GFP, the CLM-containing fluorescent proteins are not widely applied in biomedical research, and their potential as colored biomarkers is undervalued now. The CLM-containing fluorescent proteins are products of bioluminescent reactions of coelenterates. In these reactions, 'photoproteins' (complexes of polypeptides with 2-hydroperoxycoelenterazine) are recon-

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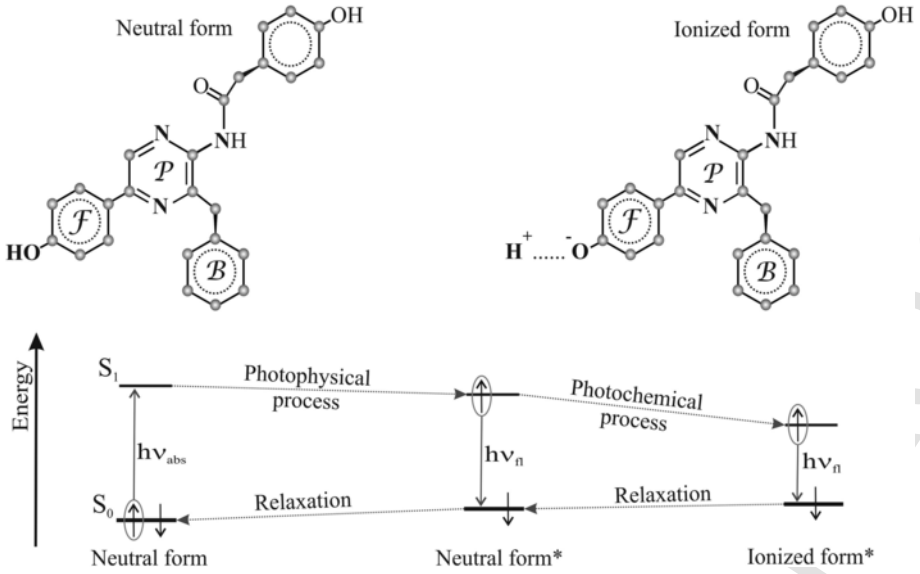

Fig. 1. Scheme of photophysical and photochemical processes in coelenteramide and coelenteramide-containing fluorescent proteins.

structed ('discharged') by addition of Ca^{2+} resulting in light emitting. This is why the CLM-containing proteins are called 'discharged photoproteins', and this term is commonly used in scientific literature.

Since the bioluminescence of photoproteins is Ca^{2+}-dependent, it is initiated in biological liquids under physiological calcium concentrations. This is a basis for medico-biological application of the photoprotein bioluminescence in different types of diagnostics: for monitoring the Ca^{2+} content, protein location in cells and tissues, and others. Additionally, bioluminescence of photoproteins is used as an intracellular marker [15,16]. These are reasons for intensive investigations of the photoprotein bioluminescence mechanism. Bioluminescence of photoprotein obelin from hydroid *Obelia longissima* is intensively studied now [7,17–19]. Genetically modified forms of obelin with different bioluminescence color have been developed [20]. Activity of upper electron-excited states of the enzyme-bound CLM in the obelin bioluminescence reaction has been demonstrated experimentally [21]; similarity to the excitation processes of other luminous organisms (bacteria and fireflies) has been discussed [21,22].

The product of bioluminescent reaction of obelin, 'discharged obelin', CLM-containing protein, is under theoretical [9–14] and experimental [17,18] investigation now. Time-resolved fluorescence experiments were conducted in [23] to determine fluorescence characteristics of neutral and ionized forms of discharged obelin. Components of complex photoluminescent spectra of discharged obelin and aequorin were studied and attributed to the different forms of CLM [24]; changes of spectral composition were analyzed under variation of calcium concentration [25], exposure to higher temperature [26,27] and exogenous compounds [28–30].

In [26,30], fluorescence spectra of discharged obelin measured under different excitation energies were analyzed. It was found that the spectra depend on the excitation wavelengths: higher energy excitation (260–300 nm) initiated fluorescence not only in the visible spectral region, but in the near UV region, too. Origination of this fluorescence is a question of special interest. It is known that low-conjugated aromatic structures can be responsible for the near UV fluorescence. In the discharged photoproteins, these might be aromatic fragments of aminoacid residues, as well as CLM aromatic fragments.

Previously [31], Eremeeva and coworkers studied UV fluorescence of photoproteins obelin and aequorin (protein-bound coelenterazine), as well as their apo-proteins. However, the UV fluorescence of the discharged photoproteins (protein-bound coelenteramide, products of the bioluminescent reactions of obelin or aequorin) has not been studied yet.

Current work aimed at characterization of UV fluorescence of discharged photoproteins and determination of moieties that might be responsible for the UV light emitting. Light-induced fluorescence spectra of three discharged photoproteins (obelin, aequorin, and clytin) at different excitation wavelengths were studied. Fluorescence spectra of CLM, fluorophore model for CLM-containing proteins, were measured in methanol and analyzed. Quantum chemical calculations elucidated peculiarities of absorption and emission spectra of CLM in methanol, and revealed molecular fragments responsible for the UV fluorescence.

2. Materials and Methods

2.1. Chemicals

Coelenteramide was obtained from NanoLight, USA; methanol, EDTA from Sigma-Aldrich, Germany; Tris from Fluka (Buchs, Switzerland). Recombinant photoproteins obelin, aequorin and clytin were produced at Photobiology Lab, IBP SB RAS, Krasnoyarsk, Russia. To obtain the discharged photoproteins, bioluminescent reactions was triggered by $CaCl_2$ in solutions of aequorin ($2 \bullet 10^{-6}$ M), obelin (10^{-5} M), and clytin ($8 \bullet 10^{-5}$ M) in 0.02 M Tris buffer and 0.005 M EDTA.

2.2. Registration of Absorption and Fluorescence Spectra

Light-induced fluorescence spectra of discharged photoproteins were recorded within 290–600 nm under 260–400 nm photoexcitation at 20 °C, pH 7.0, as soon as the bioluminescent reactions ceased. The absorption and fluorescence spectra of coelenteramide were registered in methanol solutions, $2.2 \cdot 10^{-6}$ M. The absorption spectra were measured within 250–400 nm by UVIKON 943 Double Beam UV/Vis spectrophotometer (Kontron Instruments, Italy); the fluorescence spectra were recorded by *Fluorolog 3* spectrofluorimeter (HORIBA Jobin Yvon, USA). Fluorescence spectra were cleaned

from dispersive component (i.e. Rayleigh scattering), with the scatter intensity proportional to $1/\lambda^4$ [32].

Quantum yield of CLM fluorescence (q_{CLM}) in methanol was calculated as:

$$q_{CLM} = q_s \cdot I_{CLM} \cdot A_s / I_s \cdot A_{CLM}, \quad (1)$$

with q_s being a fluorescence quantum yield of standard solution of POPOP (1,4-bis (5-phenyloxazol-2-yl) benzene), 0.75 [33]. The I_{CLM}, I_s, A_{CLM}, and A_s are integral fluorescence intensities (I) and optic densities (A) at excitation wavelengths for CLM and standard solutions (S).

2.3. Quantum-Chemical Calculations

Calculations were carried out using density functional theory (DFT) within B3LYP functional [34,35] with cc-pVDZ [36] basis by GAMESS [37] package (version Dec. 2014) in both gas phase and methanol solution. To simulate the methanol environment, two models were used: a "simple" Polarized Continuum Model (PCM) [38] and an alternative correction Solvation Model based on Density (SMD) [39]. Since the application of both models resulted in a few nanometer difference in absorption and fluorescence spectra (Supplement, Table S1) only, a conclusion could be made on equivalence of the models under the chosen conditions. The results for SMD are presented below in Section 3. The absorption and emission spectra of coelenteramide molecule appeared to be substantially dependent on excitation type (NONEQ flag in GAMESS, noneq = .t. is appropriate for vertical excitations [40], and .f. for adiabatic). The calculation was made for both vertical SMD and adiabatic SMD* excitations. The differences up to 7 nm and 15 nm contribute to the preference of SMD* for absorption and emission, respectively (Supplement, Table S1).

The CLM structure from photoprotein obelin (PDB code 1S36) was chosen as a starting geometry for the calculations to provide a comparison with protein-bound coelenteramide. Ground state geometry of coelenteramide was fully optimized in a gas phase and by SMD using B3LYP/cc-pVDZ. From the ground equilibrium geometries, absorption spectrum calculations were performed by the time-dependent (TD) DFT [41] procedure (TD // Gas phase or SMD or SMD* //B3LYP/cc-pVDZ). The excited state geometries of coelenteramide were optimized using the B3LYP/cc-pVDZ in gas phase, SMD, and SMD*. The emission energies for these geometries were obtained by TD-DFT (Fig. 2). The Cartesian coordinates of all optimized equilibrium structures are presented in Supplement. Geometries of the ground and excited states differ slightly (Supplement, Fig. S1).

The Coulomb-attenuated hybrid exchange-correlation functional (CAM-B3LYP) [42] was applied because it describes well structures with the charge transfer excited states. The geometry of ground and exciting states was optimized using SMD*//CAM-B3LYP/cc-pVDZ to get the coelenteramide absorption and emission spectra by TD-DFT. However, CAM-B3LYP showed worse results as compared to B3LYP (Supplement, Table S1, Fig. S2). The same situation was observed in [9] where the CAM-B3LYP and in [12] where the B3LYP were used. Furthermore, the "lambda diagnostic" [43] is a criterion for separating valence states from charge transfer and Rydberg states. A small value of lambda signifies a long-range excitation; a large value of lambda signifies a short-range excitation. The local excitations have a relatively large overlap, 0.45 < lambda < 0.89, indicating that the occupied and virtual orbitals involved in the excitation occupy similar regions of space. This is to be expected for local excitations, where the degree of charge redistribution is small. Our lambda diagnostic results in 0.65 < lambda < 0.75 for all calculations. This indicates that the charge transfer does not play a significant role for coelenteramide molecule in absorption and emission processes.

The overall scheme of quantum chemical calculations of coelenteramide molecule by B3LYP/cc-pVDZ method is presented in Fig. 2.

3. Results and Discussion

3.1. Fluorescence Spectra of Discharged Photoproteins at Different Photoexcitation Energies

Fluorescence spectra of discharged photoproteins were recorded at different excitation energies. Fig. 3 presents examples of these spectra at two photoexcitation wavelengths, 350 nm (A) and 280 nm (B).

The 350 nm photoexcitation initiates photoprotein's fluorescence in the visible region, Fig. 3A. In our previous studies [24–30], the visible spectra of discharged photoproteins had been approximated by a set of Gauss components. The components were found in "violet" (400–423 nm) and "blue-green" (506–566 nm) regions, and attributed to neutral and ionized forms of protein-bound CLM, respectively, Fig. 1.

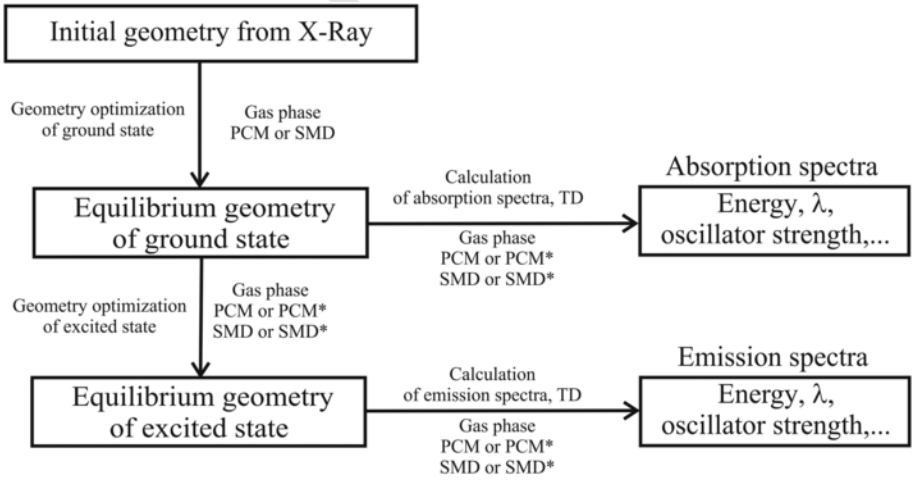

Fig. 2. Scheme of quantum chemical calculations of coelenteramide molecule by B3LYP/cc-pVDZ approach.

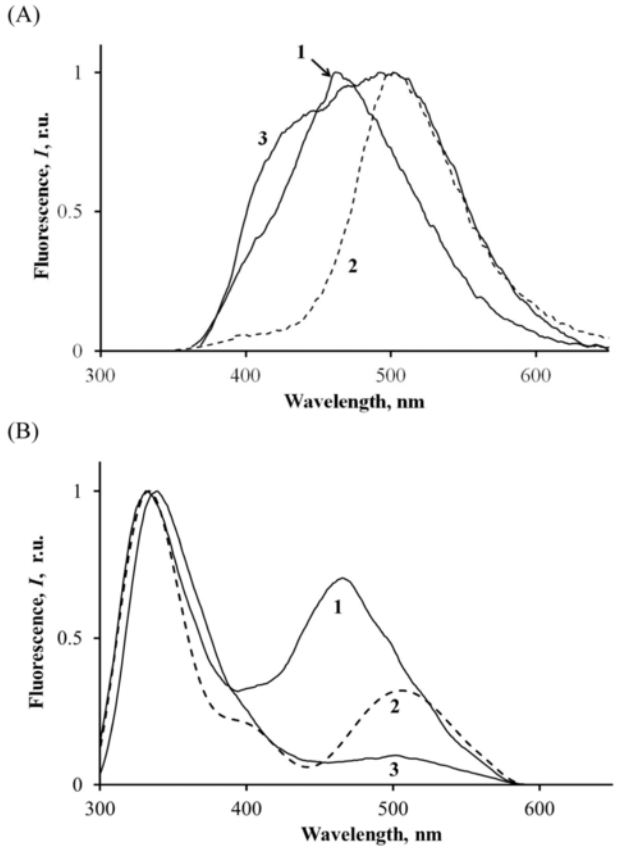

Fig. 3. Fluorescence spectra of discharged photoproteins: 1 – aequorin ($2 \cdot 10^{-6}$ M), 2 – obelin (10^{-5} M), 3 – clytin ($8 \cdot 10^{-5}$ M), at 350 nm (A) and 280 nm (B) photoexcitation.

Fig. 3B shows that UV photoexcitation (280 nm) initiates additional fluorescence peaks in the near UV region for all three discharged photoproteins. This type of UV-A fluorescence is usually attributed to spatially isolated low-conjugated aromatic moieties. In the protein-coelenteramide complex, it might include contributions of aromatic amino acid residues and CLM moieties.

To elucidate a nature of the UV component in the fluorescence of discharged photoproteins, fluorescence spectra of CLM molecule were studied at the different excitation wavelengths, and quantum-chemical calculations were applied to reveal involvement of coelenteramide aromatic fragments to its UV fluorescence.

3.2. Spectral Properties of Coelenteramide

Light-induced fluorescence spectra of CLM in methanol solution ($2.2 \cdot 10^{-6}$ M) were registered within 290–600 nm; excitation wavelengths varied from 260 to 400 nm. The 3D fluorescence spectra are presented in Fig. 4. Since the dissociation of phenolic group in methanol ($pK_a = 14$ [44]) results in a significant predomination of CLM neutral form over its ionized form (Fig. 1), the 3D spectra in Fig. 4 were attributed to the fluorescence of CLM neutral form.

Fig. 4 demonstrates that the CLM emission spectra depend on excitation energy: the excitation to the lowest electron-excited states (310–400 nm) initiates fluorescence in visible region with 420 nm maximum, while the excitation to the upper electron-excited states (260–300 nm) initiates the additional emission in UV-A region with 330 nm maximum.

Quantum yield of the CLM fluorescence in methanol at 270–340 nm photoexcitation was calculated according to Eq. (1). Its value appeared to be independent on excitation wavelength, being 0.028 ± 0.005. The quantum yield is close to that determined earlier [45] as 0.02 (methanol, 355 nm photoexcitation). Fluorescence quantum yield of discharged obelin is 0.17 as reported in [46].

Theoretical absorption (Fig. 5A) and emission (Fig. 5B) spectra in vacuum and methanol were calculated and compared to the experimental ones.

Fig. 5A shows that experimental and theoretical absorption CLM spectra in methanol are close: their maxima are 277, 294, and 332 nm vs. 264, 295, and 332 nm for experiments vs. calculations. The maxima coincide with those determined earlier in [45]. On the contrary, experimental and theoretical fluorescence CLM spectra diverged: their maxima are 330 and 420 nm vs. 318 and 397 nm, respectively (Fig. 5B). This discrepancy between the predicted and experimental values follows from inferiority of the theoretical model, which does not consider the solvent explicitly.

Fluorescence maximum of CLM in visible region (420 nm, Fig. 5B) can be compared to that of the neutral form in protein-bound

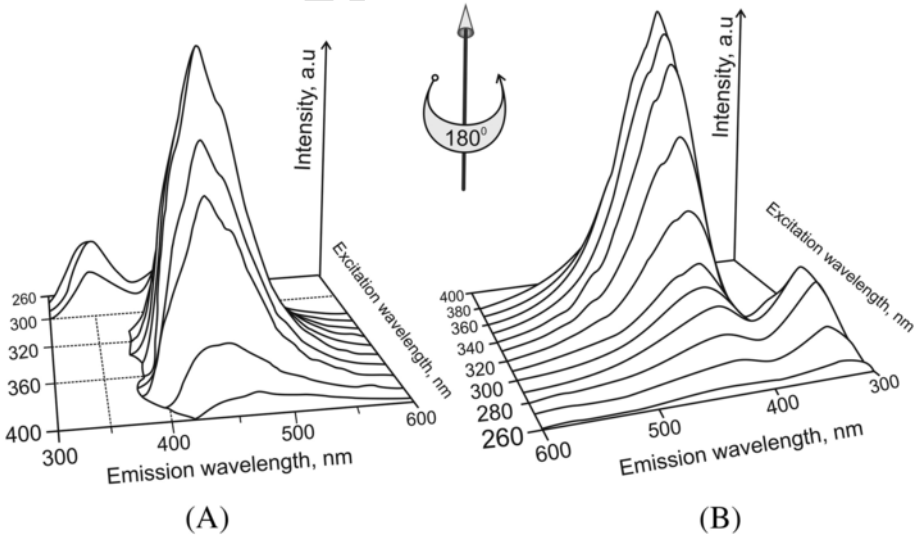

Fig. 4. Fluorescence of coelenteramide in methanol, $C = 2.2 \cdot 10^{-6}$ M. View (A) – 3D fluorescence spectra; view (B) – rotation of A through 180°.

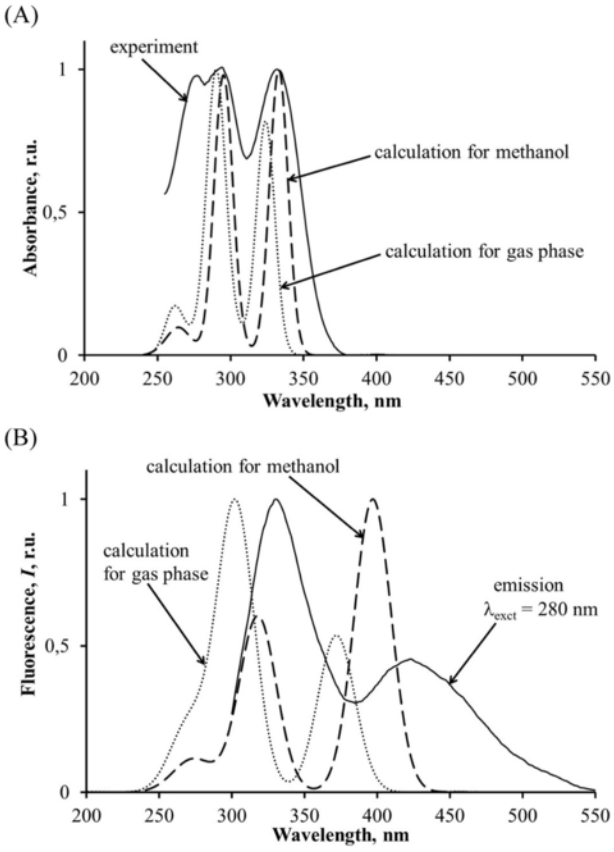

Fig. 5. Experimental and theoretical absorption (A) and fluorescence (B) spectra of coelenteramide. $C_{CLM} = 2.2 \cdot 10^{-6}$ M, methanol. Calculations were provided for gas phase and methanol by SMD*//TD//B3LYP/cc-pVDZ. Theoretical spectra were simulated by Gaussian convolution with full width at half maximum: absorption spectra of 15 nm, fluorescence spectra of 30 nm.

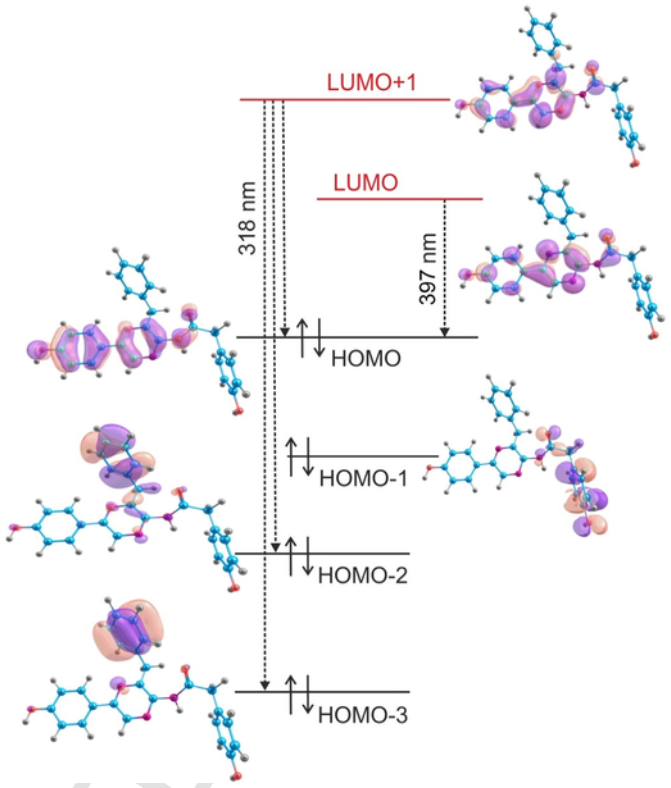

Fig. 6. Scheme of coelenteramide electron transitions and molecular orbitals involved to fluorescence. Calculation by SMD*//TD//B3LYP/cc-pVDZ.

CLM (Fig. 1). As mentioned before, the neutral form of discharged photoproteins was found in "violet" (400–423 nm) region of their fluorescence spectra [24–30].

Under higher energy excitation (280 nm, Fig. 5B), the near UV-A peak (330 nm) is observed, similar to the photoprotein fluorescence (Fig. 3B).

A description of electron transitions in CLM molecule and molecular CLM orbitals involved to the fluorescence process are given in Fig. 6. (Scheme for absorption process is presented in Supplement Fig. S3). This figure shows that long-wavelength (397 nm) peak is formed by election transition between LUMO and HOMO. Short-wavelength peak with 318 maximum is complex; it involves three transitions from LUMO + 1 to: HOMO, HOMO-2, and HOMO-3, Fig. 6.

The short-wavelength fluorescent peak of free CLM (318 nm in the predicted model and 330 nm in the experiment, Fig. 5B) as well as its electron transitions (Fig. 6) have been described here for the first time. Previous studies [9,13] considered only visible CLM fluorescence. They found that the visible fluorescence is a combination of emissions of neutral and ionized CLM forms, with the former involving electronic transitions LUMO → HOMO and LUMO → HOMO-1, and the latter involving electronic transitions LUMO → HOMO and LUMO + 1 → HOMO. All molecular orbitals involved to the electron transitions are localized at pyrazine, phenolic, and benzene fragments of CLM molecule (Fig. 6). These fragments are marked with letters *P*, *F*, and *B* in Fig. 1. It is probable that analogous electron transitions contribute to UV fluorescence of the discharged photoproteins, Fig. 3B.

4. Conclusions

We have demonstrated that photoexcitation to upper electron-excited states of CLM-containing fluorescent proteins (discharged photoproteins obelin, aequorin, and clytin) initiates emission in the near UV region, additionally to the visible blue-green fluorescence. We have found that the neutral form of CLM, fluorophore of the proteins, might contribute to this UV fluorescence. Experimental and theoretical studies have been carried out to characterize the UV fluorescence of CLM. It is probable that not only the neutral CLM form, but also the ionized CLM form, along with low-conjugated aromatic structures of the protein aminoacid residuals, might contribute to the UV fluorescence of discharged photoproteins.

This work can serve as a basis for further investigations of protein-coelenteramide interactions in the fluorescence proteins. In general, this study contributes to knowledge on internal energy transfer in biological structures – complexes of proteins with low-weight aromatic molecules. Energy migration involving upper electron-excited states in biological processes is a question of special interest. In our previous studies, we have discussed and experimentally proved involvement of upper electron-excited states of fluorophores to bioluminescent reactions of coelenterates [21] and bacteria [22,47,48]. Current study connects fluorescent activity of upper electron-excited states with structural peculiarities of low-weight biological molecules, free and protein-bound.

Hence, the study contributes to understanding of energy conversion processes in biological complexes. This field might be prospective for development of new biomaterials with specific thermo-regulative properties. Another possibility is to consider the described fluo-

rophore for activatable fluorescence. The aromatic hydroxyl group can be converted into an ester (or ether) masking fluorescence. The cleavage by an appropriate enzyme or active species (reactive oxygen or nitrogen species) would restore fluorescence [49].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jphotobiol.2016.07.004.

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